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OBSERVATIONS ON THE PATHOGENESIS OF ATHEROSCLEROSIS USING THE SERUM-LIPID INJECTED RABBIT CORNEA AS AN EXPERIMENTAL MODEL

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DEDICATION

To Dr. Levin L. Waters for his
outstanding teaching, invaluable
advice, and constant encouragement.

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I. INTRODUCTION

At present the pathogenesis of atherosclerosis is only beginning to be understood. The accumulated observations still fail to elucidate basic mechanisms and sequences of events in atherogenesis, and until these are better understood, any rational efforts aimed at prevention or modification of the disease process are necessarily limited.

In an attempt to better understand certain phases of the pathogenesis of atherosclerosis, the serum-lipid injected rabbit cornea was utilized as an experimental model in the work to be reported. The cornea was chosen because both it and the intimas of human aortas, of coronary arteries and of other elastic arteries that develop atherosclerosis are intrinsically avascular connective tissues with many structural similarities.

Before describing the experiments and results, a review of present theories in regard to certain important phases of atherogenesis and a summary of the basic anatomy, physiology, and pathology of the cornea will be presented.

II. PRESENT THEORIES IN REGARD TO CERTAIN IMPORTANT PHASES OF ATHEROGENESIS

The earliest morphological change that may be considered a forerunner of atherosclerosis in the large elastic arteries of man apparently occurs perinatally when the internal elastic lamella, the outermost part of the intima, begins to fragment.[23, 30] It splits into two or more membranes between which smooth muscle fibers appear. This new layer is called the musculo-elastic layer. With increasing age there is further splitting of the elastic membranes, the outermost one continuing to be designated the internal elastic lamella. Thus, there is progressive growth of the musculo-elastic layer of the intima.[38] This intimal thickening in the aorta is concentric and uniform. Similar but eccentric thickenings have been described in the coronary arteries of newborn infants.[22, 30, 58] Also found in the coronary arteries of newborn infants are fine extracellular deposits of lipid along the elastic fibers, in the stroma of the thickened intima, and, on occasion, in large histiocytes.[30] The question of whether the lipid first appears intra- or extracellularly, however, has not been answered. This is an important subject as it bears directly on major theories of pathogenesis. Some of these lipid deposits, it is believed, increase in size to give rise to grossly visible "fatty streaks," the next stage in the progression of the atherosclerotic plaque.

Fatty streaks are the grossly observable, superficial, yellow lines or flecks in the aortic and arterial intima of children and adolescents. Sanders[69] and Klotz[49] believed

that these fatty streaks were a manifestation of infection and not necessarily related to atherosclerosis. It is now generally accepted that fatty streaks represent a relatively early stage of atherosclerosis although no direct proof of this sequential relationship has been brought forward.

Microscopic examination of a fatty streak reveals swollen intimal connective tissue, thought to be due to the accumulation of mucopolysaccharide-rich ground substance, with an accumulation of lipid which is predominantly in large, round, lipid-laden macrophages (foam cells). Some lipid is also present in spindle-shaped cells and in cells which appear to be intermediary forms between the spindle-shaped cells and the large foam cells.[24]

McGill and Geer,[60] using the electron microscope, and Knieriem,[50] using fluorescein-labeled antibodies against myosin and actomyosin, identified lipid inclusions in the cytoplasm of intimal smooth muscle cells in fatty streaks. Thus, they showed that smooth muscle cells can be involved in intimal lipophagocytosis. McGill and Geer postulated, but did not prove, that this intracellular lipid could be the result of altered cellular metabolism due to injury and stated that as long as the lipid was intracellular and there was no connective tissue proliferation, regression of the lesion should be possible.

Belief that fatty streaks are reversible, i.e., disappear, has been expressed by several investigators including Virchow,[76] Sanders,[69] and Duff,[24] but again this has invariably been on the basis of assumptions drawn from indirect evidence. On the other hand, evidence for fatty streak

progression is the following: McGill and Geer,[60] studying fatty lesions in individuals in the third decade of life, noted that a number of these lesions contained (in addition to lipid-laden smooth muscle cells) mononuclear cells, an increased amount of connective tissue, and larger quantities of extracellular lipid. They believe that this represents the conversion of a fatty streak into a fibrous plaque. The major stimulus for fibrosis, they think, is probably the death of the foam cell with the extrusion of fatty debris into the subendothelial space.[45] <u>Since these lesions had not been followed from their inception, the conclusions are necessarily speculative</u>.

A fuller understanding of the fatty streak stage of atherosclerosis will have to await further studies on the origin and fate of its constituents, especially the lipid-containing lipophage or foam cell. Here there are many yet unanswered questions. However, the presence of lipid in the tissue macrophages of atheromatous lesions, in addition to the evidence that these cells can metabolize lipid,[32, 75] indicates that foam cells probably have a role in the deposition and/or removal of lipid in atherosclerosis. The macrophages might act in several ways in this respect: 1) they might simply take up extracellular lipid from any source and metabolize it; 2) they might synthesize and accumulate lipid intracellularly; and 3) they might ingest lipid at some site other than the vessel wall and then transport it into the arterial intima.

The fact that macrophages are able to take up cholesterol suspensions and convert them into soluble lipoproteins

has been shown by Day,[18] while French and Morris[32] have presented evidence that the macrophages can hydrolyze triglycerides. It has also been shown that the macrophages in the vessel wall and elsewhere can synthesize as well as catabolize lipid.[5, 7, 19, 94]

Leary proposed that the cholesterol in atherosclerotic lesions in both the human disease and in cholesterol-fed rabbits was carried to the arterial intima by phagocytic cells in the blood which originated in the liver and adrenal glands.[54] Gordon[37] believed that he was carrying Leary's hypothesis to its "logical conclusion" by explaining that lipid-laden macrophages, being less dense than other cells in the blood, would be on the periphery of the axial stream and would thus be the cells most able to enter the vessel wall. <u>That this issue is still controversial is indicated by the following work</u>: Simonton[73] performed a series of experiments which refuted the ideas of Leary and Gordon on the transport of lipid into experimental atheromata. Rabbits were injected with a radioisotope which had been shown to concentrate in visceral macrophages. The rabbits were then begun on cholesterol feedings. When the animals were sacrificed and the distribution of radioactivity between visceral organs and atheromata was compared, it was found that the amount of radioactivity in the atheromata was very low. Other evidence against Leary's hypothesis includes McMillan's observation[62] that there are frequent mitotic figures among the foam cells and fibroblasts of the atheromata in cholesterol-fed rabbits, thereby suggesting that the foam cells arise <u>in situ</u> by mitotic division. Also, Anitschkow had

pointed out that the presence of mononuclear cells and of all the transitional forms between them and foam cells strongly suggested that blood-borne mononuclear cells could become foam cells.[2]

Poole and Florey[67] again opened discussion of the theory that the foam cells in atherosclerosis originate from circulating lipophages with a series of beautiful electron microscope studies which showed lipid-laden macrophages passing through the endothelium overlying atheromatous plaques in cholesterol-fed rabbits. French,[31] of Florey's own group, seems to have put the above mentioned observation in its proper place by stating that

> "It is not clear, however, that migration of cells through arterial endothelium is a phenomenon of general significance."

Day,[18] too, considers the role of the foam cell in atherosclerosis to be more related to lipid metabolism than to transport of plasma lipid through the endothelial wall. In any case, the focal distribution of the lesions in atherosclerosis would seem to relegate Leary's <u>macrophage migration theory</u> to a secondary event in pathogenesis.

As the lesion advances, Duff and McMillan[24] believe, many of the foam cells disintegrate, releasing their lipid; crystals of cholesterol esters are deposited; elastic tissue fibers become frayed and fragmented; and a mass of lipid and necrotic debris results. This is the so-called atheromatous abscess.[52, 53] Surrounding this mass are foam cells, a few fibroblasts, scattered lymphocytes, and, finally, a fibrous layer which may contain finely dispersed extracellular lipid.

As the avascular intima becomes thickened, whether by local hyperplasia or by the development of atheromatous

plaques, there develops a region of relative ischemia resulting in hypoxia, which in turn acts as a stimulus for vascular ingrowth. These new vessels, according to Paterson[64] and Geiringer,[35] grow predominantly from the vasa vasorum in the outer media if the intimal thickening has occurred gradually, or predominantly from the arterial lumen if the intimal thickening has occurred rapidly, as in the deposition and organization of a thrombus. If the intima, thickened beyond a certain critical size, does not acquire an adequate blood supply, degenerative changes occur.[42]

The effect of vascularization on the fate of an intimal thickening has been a matter of discussion. Wilens,[92] who compared the vascularity of cutaneous xanthomata with that of atheromata, believes that thorough vascularization is correlated with an absence of necrosis. Other eminent investigators, including Winternitz,[93] Paterson,[64-66] and Morgan,[59] considered vascularization unfavorable because of the possibility of rupture of the poorly supported capillaries.

Paterson[65] showed intramural hemorrhages were intrinsic lesions within atheromata by demonstrating, in atheromata containing extravasated blood, no break in the overlying endothelium but instead ruptured capillaries in the plaque. On the other hand, Constantinides[15] and Friedman and Van den Bovenkamp,[34] using serial section techniques, were able to demonstrate breaks in the overlying endothelium with hemorrhage into the plaque in 55 of the 57 consecutive cases of recent coronary thrombosis studied. Obviously, the source of intramural hemorrhages is still controversial.

The integrity of a capillary wall depends on: 1) the

blood pressure within the lumen, 2) the strength of the capillary wall, and 3) the rigidity of the supporting stroma.[64] Buck[6] found that the capillaries in a thickened intima may lack a definite basement membrane. If this observation is not based on artifact due to inadequate fixation, it might be assumed that these capillaries are intrinsically weak structures. Paterson[64] believed that it was the rigid supporting stroma that was the most important factor in preserving capillary integrity and pointed out that intramural hemorrhages occurred almost entirely in necrotic, edematous plaques, the capillaries in the vicinity of large lipid deposits being greatly dilated as compared to those in nearby dense hyaline tissue. Thus, he concluded that the necrotic, edematous stroma permitted the capillaries to dilate in response to the pressure of the blood within their lumina, rupture occurring largely because of overdilatation. Wartman[77] further suggested that capillary rupture might also be caused by noxious substances in the atheromatous plaques, transient elevations of blood pressure, vigorous arterial contraction, or pressure from calcium spicules.

Theoretically, the effects of intramural hemorrhage in atheroma could at least be these: 1) sudden increase in the size of the plaque; 2) precipitation of an occluding or partially occluding thrombus by diffusion of blood or thromboplastic substances into the lumen, by ischemic necrosis of the intima, or by retrograde capillary thrombosis; and 3) acceleration of the atherosclerotic process by the accumulation of blood cells and plasma with its lipoproteins, fibrinogen, and other fractions and the subsequent degeneration

and/or organization of these. Any of the above three events might lead to organization by ingrowth of granulation tissue, which is itself susceptible to further hemorrhage. Hence a vicious cycle might be established.

Virchow,[76] in 1858, stated that the presence of fatty materials in the lesions of atherosclerosis was <u>due to a "fatty degeneration" of the intima</u>. He postulated that the pressure of the blood at certain points "irritated" the arterial intima. This led to intimal thickening which interfered with the imbibition of plasma from the arterial lumen and, therefore, compromised the normal nutrition of intimal cells. The result, he said, was fatty degeneration of the intima, implying that the lipid observed accumulated <u>in situ</u>.

Fifty years later, in 1907, Aschoff[3] discovered that the fatty material in atherosclerotic lesions was composed largely of cholesterol esters. He believed that the serum cholesterol accumulated in the intima as a process <u>secondary to some basic degenerative change</u> of the artery wall.

Anitschkow, disagreeing with this idea that the primary process was degenerative, wrote, in 1933,

> "The primany lipoid infiltration of the arterial wall gives rise to a secondary reactive hyperplasia.....The whole morphologic picture of the lipoid accumulation contradicts that idea (of primary degeneration). The normal structure of the arterial wall remains unimpaired after the lipoidal substances have made their appearances."2

However, lipid accumulation does not ordinarily occur in a normal vessel.

Meanwhile Winternitz, on the basis of morphologic studies, was advancing the <u>hemorrhage theory</u> of athero-

genesis and spoke of the late stages as follows:

> "In response to various stimuli, blood capillaries are found running through all of the coats of otherwise unchanged human vessels.....The response to injury is mediated through the capillary bed and is manifested by two more or less distinct and variably proportioned reactions: exudation and proliferation.....Hemorrhage is but an exaggerated form of exudation..... While hemorrhage, and perhaps lesser exudations are not the only source of the materials that form atheroma, they are potent contributing factors. Certainly the hemorrhagic necrosis of intimal tissue already laden with fat-filled cells results in the most extensive coalescence of lipoid materials."[93]

Later in the 1940's and in the early 1950's, Duguid[25-27,68] put forth the thrombogenic theory of atherogenesis. He believes that mural thrombi are the basis of the atherosclerotic lesion. According to Duguid such thrombi begin to organize shortly after they form but then often undergo fatty change before organization is complete, resulting in the many morphologic variations of the atherosclerotic plaque. This theory is still controversial, too, because it fails to explain the facts that: 1) thrombi are not found in arteries often enough to account for all atheromata; 2) there is little atherosclerosis in veins, where thrombi are more common; 3) there is not enough lipid in a clot to produce the gross lipid deposits of atheromata; and 4) this theory does not deal with a primary event since thrombi form secondarily to some other pathological condition.

Also prominently considered is the metabolic theory, i.e., the idea that atherosclerotic lesions are due to local disturbances of lipid metabolism. Zilversmit[94] found evi-

dence in rabbit atheromatosis that suggested that the phospholipids in atheromata were synthesized in situ rather than being derived from the plasma. However, it is questionable that the amount of any lipid fraction that can be synthesized in the arterial wall is sufficient to account for the gross deposits found in atherosclerosis.

With increasing evidence that high serum lipid levels are associated with a high incidence of atherosclerosis,[36] Page, in 1954, summarized the well-known plasma filtration theory of atherogenesis:

> "This filtration concept is based on the view that atherogenesis is due to the tissue reaction to substances filtered from plasma as lipoprotein by lateral arterial pressure, and deposited in the intima as 'foreign' lipid. Most of the filtered materials pass on harmlessly to be picked up by the adventitial capillaries or the lymph. But some may stay behind, whether because the vessel fails to function properly as a filter or because the size, shape and charge of the lipoproteins is such as to allow them to stick.
>
> "Perhaps the most important point which needs emphasis is the basic similarity in initial relative composition of vessel wall and plasma lipid. This supports the view that the source of the vessel wall lipid is plasma lipid. The beta lipoprotein is probably the chief carrier in plasma of unstably held lipid which would be shed within the vessel as the filtered lipoprotein breaks down. This lipid is then 'foreign' and the nidus of a foreign body reaction."63

In 1956 Hanig et al.[39] extracted beta lipoproteins from atherosclerotic human aortas. Watts' later work[91] with specific fluorescent antibodies, which enabled him to demonstrate serum lipoproteins in the arterial wall, and the work of many others supported the following theoretical mechanism for atherogenesis: plasma, containing beta lipoproteins which act as carriers for lipids, perfuses the arterial wall and enters

intimal cells. Because of inadequacies in oxidative metabolism of these lipids within cells, the lipids accumulate, and foam cells are thus formed. When the foam cell dies it releases its lipid into the intersticies of the vessel wall. As the process repeats itself many times over there is an inflammatory reaction with a scar ultimately forming. The result is an atherosclerotic plaque.

The plasma filtration theory, however, fails to mention, much less account for, the focal nature of the lesions of atherosclerosis, a disease process in which certain sites are typically involved. Indeed, if the plasma filtration theory were in itself a complete explanation of atherogenesis, a diffuse, gradually progressive, uniform, atheromatous change might be expected in all of the large vessels of all persons. This is obviously not the case.

It has been shown by many investigators that _injury_, either physical or chemical, to the vessel wall in the experimental animal is certainly _a factor in the localization of arterial lesions_ in hypercholesterolemic animals. In 1914 Anitschkow[1] demonstrated the deposition of lipid in the aortas of rabbits fed cholesterol (hyperlipemia) and given epinephrine (arterial injury). Later Schlichter _et al._[70] cauterized the ascending aorta of cholesterol-fed dogs and in some animals found fatty deposits at the site of injury. Kelly _et al._[48] froze the abdominal aortas, renal arteries, and iliac arteries of cholesterol-fed rabbits and produced atheromatous localization at the sites of injury. Waters showed that particles from the blood, such as methyl cellulose[79] or plasma lipoproteins,[20, 21, 80, 81, 85, 88]

when given to allylamine-treated dogs[78] in doses productive of only minimal arterial lesions in normal dogs, cause massive foam-cellular lesions in the coronary arteries selectively at the sites of allylamine injury. Thus, injury, whether physical or chemical, causes increased permeability of the vessel wall to serum lipoproteins and, in hypercholesterolemic, hyperlipoproteinemic animals, a marked tendency for lipophagic lesions to form at the sites of injury.

> "The selective localization of lipids in the artery walls at areas of injury emphasizes the dependence of this phenomenon on the presence of local increase in permeability."[85]

The significance of the injury--inflammation--increased permeability component of atherogenesis has been summarized by Waters:

> "In our laboratory the hypothesis followed has been that the atherosclerotic lesion is basically an inflammatory reaction of vascular tissues to injury, an inflammatory reaction greatly modified by the anatomy and physiology of these specialized structures and by their contact with the circulating blood, as well as by the intensity and duration of the stimulus. A central feature of arterial inflammation as related to atherosclerosis is increased, transient, local permeability of the vessel wall to blood plasma. Early elements of the inflammatory reaction are fluid exudates in the vessel wall with the deposition of lipid substances. As the lesions progress, lipophagocytic mononuclear, smooth muscle, and fibroblastic cells accumulate. Lipid is removed both intra- and extracellularly and the lesions progress to scar. With time and repetition of events, the intimal scar becomes larger and denser and its nutrition may be impaired, with resultant central autolysis. There is then secondary exudation, further lipid deposition, and again lipophagic cells accumulate and enlarge the fatty, granulomatous lesion. The stimulus of prolif-

> erating connective tissue may now elicit ingrowth of vasa vasorum, increasing the capabilities of the focus for still further inflammatory events. Vascularization of the plaque, for example, may lead to increased cellular exudation with proteolytic enzyme release, or frank hemorrhage. These processes set the stage for ulceration, thrombosis, calcification, or aneurysm formation."90

A good deal of the present knowledge of atherosclerosis has been gained from experimental work with animals. One of the most useful animal models is the cholesterol-fed rabbit. Supplementation of a rabbit's diet with one-half to one gram of cholesterol solubilized in oil per day induces a hypercholesterolemic, hyperlipemic state in a high percentage of animals. Within hours or days after the increase in blood lipid levels, lipid begins to appear in focal regions in the intima of the arterial system in extracellular ground substance, in histiocytes, and in fibroblasts. Foam cells accumulate; elastic laminae may show degenerative changes; and fibrosis occurs.[2, 24]

As Anitschkow wrote:

> "The great morphologic similarity of the atherosclerotic connective tissue plaques in experimental rabbits on the one hand, and in human atherosclerosis on the other, is a matter of very considerable significance since it constitutes experimental proof that the primary lipid infiltration of the arterial wall gives rise to a secondary reactive hyperplasia of the fibrous-elastic elements, resulting in the formation of typical 'sclerosis' plaques of a connective tissue character."2

That <u>this so-called "experimental proof" of lipoid infiltration is still very controversial</u> is clearly shown by the efforts already cited to demonstrate disturbances in lipid metabolism in the vessel wall as the underlying mechanism of

atheroma formation.

Differences between the lesions in cholesterol-fed rabbits and in human disease, however, are present. In the rabbit lipids accumulate in large amounts not only in the arterial intima but also in the reticulo-endothelial system, mesenchymal tissue, and parenchymatous organs; vascularization and atheroma formation of the intimal lesions are not prominent; and there are differences in lesion distribution.[24]

Constantinides[14] developed an animal model for the late stages of atherosclerosis, i.e., plaques with overlying thrombi, by intermittent cholesterol feedings of rabbits over a two-year period followed by injections of norepinephrine to produce arterial injury and of Russell Viper Venom to produce a hypercoagulability of the blood. But *this model, like the cholesterol-fed rabbit, permits systematic analysis neither of the initial constituents of the lesion nor of the exact sequence and timing of events that follow*.

III. BASIC ANATOMY, PHYSIOLOGY, AND PATHOLOGY OF THE CORNEA

In 1942 Mann and Pullinger[56] noted that chronic mustard gas lesions of human corneas led to the deposition of cholesterol and other lipids, and they suggested a similarity between the deposition and ultimate fate of lipid in the avascular cornea and in the avascular arterial intima. This observation was noted and followed up by Waters, but before his studies are presented, a discussion of basic corneal morphology, physiology, and pathology is needed.

The cornea consists of five layers beginning anteriorly: 1) stratified squamous epithelium; 2) Bowman's membrane, which merges with the superficial layer of the stroma; 3) substantia propria, or stroma, which consists of connective tissue cells and broad bands of interlacing collagenous fibrils arranged parallel to the surface of the cornea; 4) Descemet's membrane, a secretory product; and 5) endothelium. The cornea is avascular. The limbus is the peripheral region which forms a transition zone between the cornea and the conjunctiva-sclera. Its stroma is composed of irregularly arranged lamellae and is rich in blood vessels.[33]

The remarkable transparency of the cornea has been explained by comparing the cornea to a diffraction grating, the collagen fibers representing the scratches of the grating. Interference suppresses the scattering of light in any direction other than that of the incident beam, and the cornea, therefore, appears transparent. This hypothesis requires that all the fibrils be of uniform and equal diameter and be regularly arranged.[16, 57]

Hydration of the cornea also deserves consideration. Since the cornea is permeable to water and the major ions of tears and of aqueous humor,[40] it may be assumed that the cornea, whose fibers are only 50% hydrated,[57] actively excretes fluid. But both the epithelium and the stromal cells are poor in endoplasmic reticulum and mitochondria.

> "Only the endothelium, which appears to be the main pump of the cornea, has a high enough concentration of mitochondria to suggest that these cells are doing much more than just maintaining themselves."44

Experimentally the cornea has been used for a number of years to study neovascularization. The advantages of using the cornea for this stem from the facts that 1) the cornea is easily observed in the living experimental animal; 2) the cornea is normally transparent and so changes in any of its layers are obvious; 3) it is relatively homogeneous; and 4) vascularization can be induced without the formation of much granulation tissue.[8, 47]

Vascularization of the cornea may be induced in many ways.[4, 8, 28, 33, 46] The final stimulus to neovascularization is probably one or more of the following: 1) elaboration of a substance which stimulates vessels to grow towards the site of its maximum concentration; 2) destruction of a growth-inhibiting substance; and 3) reduction of the compactness of the tissue which had previously prevented vessel ingrowth.

Ashton and Cook[4] carefully studied the effects of extracts of vascularizing corneal tissue on normal corneas and were able to find no evidence for the first two above mentioned mechanisms, but they did regularly produce vasculari-

zation when they gave repeated injections of saline into the cornea near the limbus. This

> "group of experiments indicates that a reduction in the compactness of the normal corneal tissue, whether produced mechanically or by the induction of corneal oedema, may give rise to vascularization, providing the potential pathways resulting from the stromal separation communicated with the limbal vessels."[4]

A possible reason for the decrease in corneal compactness leading to vascularization is suggested by the work of Langham.[51] He noted that the cornea is normally in a state of relative hypoxia, itself a stimulus to vascularization. Thus, once the compactness of the cornea is reduced, as by edema, vascularization may occur in response to the relative hypoxia.

Cogan[8] made a detailed study of the actual sequence of events in the vascularization of the cornea. He induced vascularization by injuring the rabbit cornea with either heat, acid, or alkali. He found that the first change in the limbal vessels was a local engorgement of capillaries and venules. These vessels then either formed buds or went on to develop saccular aneurysms, the buds and aneurysms being predominantly on the side of the vessel toward the lesion. On approximately the fourth day the aneurysms burst with the resulting microhemorrhages radiating out in a spicule-like fashion. The laminar structure of the cornea probably was the cause of the spicule-like arrangement of the hemorrhages. Two days later new capillaries were seen in the regions of the hemorrhages. A similar sequence has been observed by Julianelle and Bishop[47] and by Friedenwald.[33]

In regard to the formation and subsequent rupture of the microaneurysms of ingrowing corneal vessels, Cogan concluded:

> "The only reasonable explanation would appear to be that the distention and bursting are due to a decrease in the external pressure through lessened support on the wall of the blood vessel, rather than to an increase in the intravascular pressure.....Swelling of the cornea allows the tissue spaces to become loose; when the cornea is swollen, there is less support for the blood vessel wall. The thesis is therefore offered that the events in new vessel formation in the cornea are initiated by reduction in the tissue compactness of the surrounding stroma."[8]

However, if corneal edema is to be assumed a prerequisite for corneal vascularization, it is difficult to explain the naturally occurring vascularization in the cornea of newborn lambs of Rocky Mountain bighorn sheep. Their corneas vascularize at about the time of birth and then devascularize a short time thereafter. During this entire period the cornea remains clear[74] and, therefore, one may conclude, nonedematous.

Cogan has also made a study of lipid in the cornea.[9-13] Starting with naturally occurring human keratopathies in which lipid is a prominent feature, Cogan[9] noted that in arcus senilis there is a diffuse extracellular sudanophilia of the peripheral cornea and sclera. Foam cells do not occur. Between the cornea and sclera is the relatively lipid-free zone of the vascular limbus. Also, fortuitous ingrowths of blood vessels are accompanied by a corresponding deflection of the arcus. Since de-lipidized sections of the corneas with arcus show no abnormalities, degeneration can not be considered an underlying cause of the arcus.

Lipid keratopathy, an entity characterized by the appearance of fat in a region of prior vascularization (usually due to a previous non-specific keratitis), presents a much different picture than does arcus. Here lipid is both extra- and intracellular.

> "The granular sudanophilia consists of granules situated extracellularly and superimposed on a collagenous framework in areas of relative acellularity (necrosis). The globular sudanophilia on the other hand predominates in areas of increased cellularity and the globules are situated chiefly within the cells. The areas of granular and globular sudanophilia tend to be mutually exclusive and the conclusion is inescapable that the initial change is globular, intracellular fat formation that becomes converted into a granular extracellular distribution with necrosis of the tissue."[9] (underlining, L.E.G.)

Cogan extended his discussion of granular and globular lipid to the human arterial intima:

> "The portion of the intima adjacent to the lumen, and to a less extent the portion adjacent to the media, tends to be relatively acellular and contains the granular type of lipid. This is consistent with the suggestion that the granular sudanophilia is derived from the globular sudanophilia as the tissue becomes necrotic."[13] (underlining, L.E.G.)

A third and final keratopathy is that found in cholesterol-fed rabbits. This is a type of arcus which is more like human lipid keratopathy than human arcus. It is associated with considerable vascularization, and the lipid is mostly intracellular,[9] a picture very much like the atheromatosis of cholesterol-fed rabbits.

In 1955 it was noted[82, 83] that the injection of clear (chylomicron-free), normal dog plasma into the center of the dog cornea gave rise to no lipid deposit: injection of clear,

normal human plasma or serum led to a sudanophilia with lipophagocytosis in the stroma; and injection of dog or human chylomicrons resulted in a lipid-rich, foam-cellular lesion with only minor inflammatory conponents. There was no vascularization. Waters[84] also observed that if the concentrated serum lipids were injected into the periphery of the dog or rabbit cornea, vascularization of the lipid plaque did occur. This was accompanied by edema, cellular exudation, more massive foam cell accumulation, acceleration of lipid removal, and in some cases necrosis, hemorrhage, and ulceration. Similar results have since been obtained by Silver, Weigensberg, and McMillan.[72]

The close resemblance of the experimental serum-lipid injected corneas to human atherosclerotic lesions was appreciated,[86, 87] and the reaction of the rabbit cornea to the injection of 0.1 ml. of homologous serum from cholesterol-fed rabbits was tested. It was found that serum cholesterol levels of 120 mg. per cent or higher regularly produced the lipophagocytic reactions mentioned above.[89]

Thus, work with the corneal model up to the present has outlined certain morphological parallels between the serum-lipid injected animal cornea and the lesions of human atherosclerosis. The present study utilizes the corneal model to investigate systematically several major and as yet controversial points in the pathogenesis of atherosclerosis. These are:

1) questions of extracellular versus intracellular lipid in the initiation of atherogenesis;

2) questions relating to the biology of lipophages throughout atherogenesis;

3) questions in regard to the role of vascularization and hemorrhage in the development of the lesion;

4) questions relating to the quantity of serum lipids associated with atherogenesis;

5) questions relating to collagen changes in developing atheromata; and

6) questions relating to the cumulative effects of repeated infiltrations of connective tissue by lipid-rich and lipid-poor serum.

IV. EXPERIMENTAL

A. MATERIALS AND METHODS

New Zealand white rabbits of either sex, weighing 3-5 kg., were anesthetized with intravenous pentobarbital (25 mg./kg.) for corneal injections. Injection of 0.1 or 0.3 ml. of normal or hypercholesterolemic, hyperlipoproteinemic, homologous rabbit serum or of other pertinent materials was made tangentially into the cornea near the limbus through a sterile 30 gauge hypodermic needle inserted bevel up. The resultant plaque was 8-15 mm. in diameter, and its edge was in contact with the limbus. Before and after each injection the eye was gently irrigated with one per cent boric acid solution. There were no cases of infection.

Serum was obtained by aseptic technique from normal rabbits, and an aliquot of each sample was analyzed for lipids and protein. Serum lipid fractions were determined by the following methods: cholesterol and cholesterol esters--modified Schoenheimer-Sperry;[71] lipid phosphorus--a modified Youngburg procedure;[41] and fatty acids--a modified Stoddard and Drury technique.[55] Average serum levels were total cholesterol 35 mg. %, free cholesterol 7.5 mg. %, fatty acids 11 meq./l., phospholipids 4 mg. %, and total protein 7 gm. %. Hypercholesterolemic, hyperlipoproteinemic rabbit serum was obtained from cholesterol-fed rabbits and averaged total cholesterol 1,000 mg. %, free cholesterol 500 mg. %, fatty acids 80 meq./l., phospholipids 16 mg. %, and total protein 7 gm. %. The serum was examined microscopically to rule out the presence of chylomicra. Comparable

serum from other cholesterol-fed rabbits was shown by Gofman *et al.*[36] to have high levels of S_f 10-30 class lipoproteins. This group of lipoproteins in the rabbit corresponds to the S_f 10-20 lipoproteins regularly found to be significantly elevated in patients with known coronary atherosclerosis.[36]

Animals were maintained in individual cages in clean laboratory conditions, were fed a standard rabbit chow (Purina Mills, Inc., fat content 2.0%), and were given drinking water *ad libitum*. For histological studies animals were sacrificed by intravenous pentobarbital anesthesia. In some cases the eyes were then perfused with 1% India ink in 0.9% saline at 100 mm. Hg pressure in order to demonstrate fully the capillaries in the cornea. The corneas were removed and cut in such a way as to divide the lesion into two approximately equal parts. One half of each cornea was fixed in 10% formalin, the other half in Friedenwald's fixative (two parts of saturated aqueous solution of $HgCl_2$ plus one part of absolute alcohol for 48 hours followed by a change to 80% alcohol). The formalin-fixed tissues were cut by frozen section technique and stained by Sudan IV. Paraffin sections were made of the adjacent Friedenwald-fixed tissues which were stained with hematoxylin and eosin. Selected sections were also stained by toluidine blue, Masson, or von Kossa's stain for calcium.

Multiple microscopic sections of aortas and coronary arteries from approximately 20 patients with and without significant atherosclerosis were also studied, and a careful comparison of the human lesions and the experimental corneal plaques was made.

B. RESULTS

1. The Natural History of the Vascularizing Corneal Lesion that Follows Injection of Lipoprotein-Rich Homologous Serum

Microscopic examination of control normal rabbit corneas revealed a dense, uniform, collagenous, avascular stroma with interspersed elongate stromal cells. There was no sudanophilic material.

Injection of 0.1 or 0.3 ml. of sterile physiological saline solution led to the immediate production of an edematous, opaque, white plaque which grossly cleared completely within 24 hours. Microscopic examination of the plaque immediately after injection revealed it to be an edematous region of separated collagen fibers. By 24 hours the edema had largely subsided, the collagen fibers being less separated from each other. These changes continued to regress, and by the end of two weeks there was no evidence of edema. A small scar from the needle tract, often with epidermidization of the tract, persisted. There was no sudanophilic material or vascularization.

Injection of 0.1 ml. of chylomicron-free, hypercholesterolemic, hyperlipoproteinemic, homologous serum into 30 corneas led to the immediate production of an edematous, opaque, yellow-white plaque. This plaque, which began to clear a few hours after injection, returned to its original opacity grossly within several days. By 7-10 days many fine vessels could be seen growing into the plaque from the limbus.

Approximately two weeks after injection, in most animals there was a narrow band of clearing of the cornea paralleling the limbus where contact had previously been made between it and the plaque. By this time there was commonly also a narrow zone of clearing around some of the vessels penetrating the plaque. Vascularization progressed for the next 2-3 months, by which time the plaque was usually separated from the limbus by a clear zone 2-3 mm. wide. The plaque was less dense than originally, often having a mottled appearance associated with the clearing around the vessels in the plaque. After this stage, the vessels gradually became smaller and fewer grossly. At the end of one year the plaque was greatly reduced in size, opacity, and vasculature, but nevertheless, persisted.

Microscopic examination of the plaques produced by the injection of 0.1 ml. of chylomicron-free, hypercholesterolemic, hyperlipoproteinemic, homologous serum revealed the following: At one day there was edema in the region of the plaque. The collagen fibers were somewhat frayed, swollen, spread apart, and pale. There was a very finely granular, extracellular sudanophilic material apparently situated on the surfaces of collagen fibers.

At four days lipophagic mononuclear cells were first noted. They were small and few but were uniformly distributed throughout the plaque, giving the impression that they arose, at least initially, *in situ* rather than from circulating cells coming from the limbal vessels.

At six days foam cells were larger and more abundant. The lipid they contained was predominantly globular in distinction to the very fine granules that were still present

extracellularly at the surfaces of collagen fibers. Collagen swelling was prominent. Dilated capillaries could be seen growing into the plaque from the limbus and were accompanied by an infiltrate of polymorphonuclear leukocytes, monocytes, some lymphocytes, and a few plasma cells. In some cases there were microhemorrhages at the advancing tips of the capillaries.

At three weeks the foam cells were no longer uniformly distributed in the plaque. Instead they were most numerous in the edematous portion of the plaque which had not yet become vascularized and were least common in the non-edematous portions of the cornea surrounding the plaque and in the para-limbal zone. The latter corresponded to the grossly cleared portion of the plaque.

At four weeks the swollen, edematous region of the plaque persisted. Almost all of the lipid was globular and within foam cells which were even larger than before. The cellular infiltrate at the limbus was greatly reduced and was predominantly mononuclear. Vascularization progressed, and many vessels were seen parallel to the collagen lamellae, often giving off branches that crossed these lamellae. Foam cells were most prevalent in the region where the collagen fibers appeared frayed but could also be seen in the regions of the swollen, smudged-appearing collagen adjacent to the plaque.

By two months the plaque was entirely vascularized, and foam cells were most numerous near the blood vessels, giving the impression that many of these mononuclear cells had been brought to the plaque by the ingrowing vessels.

At one year the foam cells were smaller and fewer but were still present, as were the altered, swollen collagen bundles.

2. The Effects on the Cornea of Injection of an Increased Amount of Lipoprotein-Rich Serum

In an attempt to accelerate and augment the changes noted above, larger plaques were produced by the injection of larger amounts (0.3 ml.) of chylomicron-free, hypercholesterolemic, hyperlipoproteinemic rabbit serum. This procedure was carried out in eight corneas. It resulted not only in a larger plaque and more rapid and intense vascularization, but also in a more marked foam-cellular response and more prominent degenerative changes in the collagen fibers. The production of so many large foam cells provided an opportunity to study the pleomorphism of these cells. Spindle-shaped cells and all the intermediate forms up to and including engorged, round, lipid-filled foam cells were seen (as is the case in human atherosclerosis[24]), giving more support to the hypothesis that some of these cells arise, or at least mature, *in situ*.

3. The Effects of Repeated Injections of Lipoprotein-Rich Serum

To test the effects of repeated deposits of serum lipids and to stimulate the process still further, repeated injections of 0.3 ml. of the hyperlipemic rabbit serum were made into the same cornea. Up to five injections of 0.3 ml. were given

successfully at weekly intervals into 16 corneas. Most of the animals were sacrificed 2-3 months after the last injection, thus allowing the response to the acute injury of injection to subside and permitting a large foam-cellular response to occur. These corneas were grossly swollen and had large, yellow, well-vascularized plaques. Microscopically, intensely foam-cellular, thoroughly vascularized lesions were evident. The characteristic clefts of cholesterol ester crystals, a regular feature of late human atherosclerosis, were common, and the tissues gave a positive Schultz test (based on the Liebermann-Burchard reaction[17]) for cholesterol. The collagen in all cases was pale and swollen and in some cases also frayed and fragmented. The decreased number of cell nuclei in these regions indicated that there was some degree of tissue necrosis. In those instances in which there was fragmented collagen, large numbers of lipophages were always seen in the immediate vicinity of this changed collagen, suggesting that these foam cells were apparently ingesting the swollen, degenerating, lipid-impregnated collagen fibers. Again, microhemorrhages were seen near the advancing capillary tips.

4. The Effects of a Single Injection of Normal, Lipoprotein-Poor Serum

A series of 14 rabbit corneas was studied after a single injection of 0.3 ml. of homologous normal rabbit serum. The lipoprotein content of this serum was: total cholesterol 35 mg. %, free cholesterol 7.5 mg. %, fatty acids 11 meq./l.,

phospholipids 4 mg. %, and total protein 7 gm. %. This produced an opaque white plaque immediately. Over the course of the following five days the plaque cleared completely. There was no vascularization. Microscopically, six out of six of the edematous, normal rabbit-serum injected corneas showed no sudanophilia for the first few days, but four days after injection, when the edema had decreased considerably, there was a trace of sudanophilic material at the surfaces of the collagen fibers. This became slightly more prominent during the following week as the edema further subsided, suggesting that much of the serum water was being removed while the lipid was remaining and was being concentrated in the cornea in association with collagen fibers. This sudophilia was noted in three of the eight corneas into which the serum had been injected at least four days prior to sacrifice. Neither a foam-cellular response nor vascularization could be demonstrated.

5. The Effects of Repeated Injections of Normal, Lipoprotein-Poor Serum

The above observations led to an investigation of the effects of repeated injections of normal rabbit serum into the same cornea. First a group of control corneas was prepared and examined. The six control eyes received repeated intracorneal injections of 0.3 ml. of sterile physiological saline solution. Within 24 hours of each injection the plaque had grossly cleared completely. Four such injections at four day intervals, followed by two months in which no further injections were given, led to the growth of a few

tiny blood vessels into the region of the transient plaques. There was no sudanophilia or lipophagocytic response. Then the experimental group of four rabbit corneas was prepared. Each of these received four injections of 0.3 ml. of homologous normal rabbit serum (total cholesterol 35 mg. %) at four day intervals followed by two months in which they received no injections. Each injection produced a white edematous plaque, but within a few hours the plaque became much less opaque and swollen. In these corneas the plaques never completely cleared; they persisted as faint, circular, hazy regions. Several small vessels could be seen growing into the plaques from the limbus. Microscopic examination of these plaques revealed, in three of the four corneas, a lesion consisting of dilated and congested capillaries, swollen collagen, definite foam cells containing globules of lipid, and a sparse mononuclear infiltrate.

V. DISCUSSION

The most prominent histologic features of human atherosclerosis and of the vascularizing serum-lipid injected rabbit cornea have been presented. These two processes have striking similarities. Both the normal human arterial intima and the normal rabbit corneal stroma are avascular connective tissues, rich in mucopolysaccharides and collagenous connective tissue in lamellar arrangement. Both are near vascular beds capable of proliferation and expansion into the adjacent avascular tissue: the medial vasa vasorum into the intima of the artery and the limbal vessels into the cornea.

An obvious difference between the artery and the cornea is that the former is continually subjected to pulsating intra-arterial pressure with but a single layer of endothelium between the arterial blood and the avascular intimal connective tissue. However, assuming that vascular injury in man occurs throughout life and leads to periods of increased intimal permeability, thereby permitting even the large lipoprotein molecules of the serum to perfuse the intima, the parallel between human atherogenesis and the corneal model in the experimental animal can be preserved. This is done by injecting directly into the corneal stroma homologous serum from hypercholesterolemic, hyperlipoproteinemic donor animals. Naturally it must be borne in mind that the corneal stroma _is_ _not_ arterial intima and that evidence from _any_ model is only indirect.

Beyond this, however, the advantages of the corneal model from the standpoint of controlled observations are

many: the exact time and duration of the lipid-connective tissue reaction are known; the precise amount of lipid deposited in the connective tissue can be calculated; the lesion can be carefully followed grossly and particular attention paid to the events of vascularization and lipid removal without in any way disturbing the process; and additional lipid-rich serum can be added repeatedly to the developing lesion.

Since the sequence of events in the experimental model could be confidently related to the initial constituents of the injected serum and to their subsequent interactions, evidence bearing on some of the controversial questions concerned with the pathogenesis of atherosclerosis was obtained. The results have to do with the following little-understood subjects in the evolution of the arteriosclerotic plaque: 1) the nature and site of appearance of the initial lipid in the arterial intima, 2) the role of the foam cell, 3) the mechanism and local effects of intramural hemorrhage, 4) the so-called "atherogenic" serum lipid levels, 5) the mechanism and significance of collagen damage, and 6) the cumulative effects of repeated episodes of vascular injury.

The corneas into which chylomicron-free, hypercholesterolemic, hyperlipemic rabbit serum had been injected and the animals serially sacrificed, demonstrated these points: First, lipoprotein-rich serum introduced into an intrinsically avascular connective tissue such as the cornea, but within a few millimeters of blood vessels, impregnates and leads to swelling of collagen fibers almost immediately and elicits a lipophagocytic response in four days. These early lipophagic

cells are apparently modified connective tissue stromal cells. Second, within six days after the introduction of the lipid-rich serum into the connective tissue, many dilated capillaries are already invading the swollen, edematous, previously avascular connective tissue. Microhemorrhages are not unusual. Third, by the end of a month almost all of the lipid, originally present in serum in molecular solution and shortly thereafter seen to be impregnating collagen fibers as fine extracellular granules, is within large foam cells. By this time, also, some lipid, especially in the zone nearest the greatest number of blood vessels, i.e., the para-limbal region of the cornea, has been removed. In addition, the acute inflammatory cellular exudate seen shortly after injection becomes wholly mononuclear within a month. Fourth, by the end of the second month both the foam-cellular response and vascularization reach their peaks, and there is the suggestion that some of these foam cells originate from mononuclear cells brought into the plaque by the invading vessels. Fifth, one full year after the injection of lipid-rich serum into the normally avascular cornea, lipid-filled foam cells, vascularization, and damaged collagen, although quantitatively decreased from their levels at two months are still unquestionably present.

Thus, from corneas injected once with homologous hyperlipemic serum it can be seen that lipoprotein in its native serum, when placed in an avascular connective tissue, first impregnates collagen fibers as very fine granules and is later taken up into lipophagic cells where it assumes a globular form. It should also be noted that at no time

during the full year of observation were foam cells seen to be degenerating or otherwise breaking down and releasing their lipid content. Once lipid is phagocytosed it remains intracellular for at least a year. This is further demonstrated by the fact that once regions of established corneal plaques clear, opacity does not recur. This sequence of events relating to the fate of stromally deposited serum lipoprotein is directly opposite to the speculations of Cogan[9, 13] and others that lipid in an avascular connective tissue appears first as intracellular globules and later becomes extracellular fine granules as the lipid-containing cells degenerate. These observations also cast doubt upon Leary's theory of lipophage migration into the intima by supporting the hypothesis that molecularly dispersed serum lipoproteins can be retained in avascular connective tissue and can be progressively incorporated into lipophages. Observations of the corneal model also present strong alternate evidence against the theory that the lipid in an atheroma is either derived primarily from cellular metabolic derangements (fatty degeneration) as postulated by Virchow,[76] McGill and Geer,[60] and others or synthesized <u>in situ</u> as suggested by Zilversmit[94] and Holman.[43]

As noted by Ashton and Cook[4] in corneas vascularizing during maintained edematous states and by Cogan,[8] Julianelle and Bishop,[47] and Friedenwald[33] in corneas vascularizing after physical or chemical injury, microhemorrhages are common in the vicinity of advancing capillary tips. This regular association of neovascularization and microhemorrhages is also seen in the rabbit corneas injected with

chylomicron-free, hypercholesterolemic, hyperlipoproteinemic serum and should be considered as a possible mechanism for hemorrhage into atheromatous plaques.

One respect in which corneas singly injected with hyperlipemic rabbit serum might fail to parallel human atherosclerosis is that in man there is undoubtedly more than one episode of lipid infiltration into the arterial wall at a given site during a lifetime. Each episode of vascular injury with its attendant increase in intimal permeability may lead to an accompanying increased perfusion of the intimal connective tissue by beta lipoprotein macromolecules. Such repeated injury with subsequent repeated exposure of the intima to beta lipoproteins was simulated in the cornea model by giving sequential injections of lipid-rich serum into the same cornea. This procedure results in similar but more intense lesions than does a single injection. All aspects--foam-cellular reaction, vascularization, and collagen damage as evidenced by swelling, fraying, and fragmentation of the fibers--are pronounced. In addition, a regular feature of these large plaques is that the most marked foam-cellular response is in the immediate vicinity of the frayed, swollen, lipid-impregnated collagen, suggesting that these foam cells may be taking up the degenerating, lipid-impregnated collagen fibers. Morphologically identical zones of swollen, degenerating collagen fibers and lipophages are seen repeatedly in human atheromata. Also common to both the serum-injected corneas and human atherosclerotic plaques are the regularly seen clefts of cholesterol ester crystals. Noteworthy, too, is the fact that,

in the case of the large plaques, fibrosis does occur, beginning within three months of the initial serum injection. Thus both the fatty streak and the fibrous plaque stages of atherosclerosis have been closely simulated in the cornea. From the first introduction of lipid-rich serum into avascular tissue, the lesion progresses to a fatty streak and then to a fibrous plaque, all within a period of three months, thus establishing a definite sequence and timing of events in a more controlled fashion than even any experimental intra-arterial model.

A question which invariably arises in a discussion of atherogenesis either in man or in animals is precisely what is the minimal serum lipid level which is associated with the establishment of atherosclerotic lesions. The experiments in which homologous normal rabbit serum (cholesterol 35 mg. %) was injected into rabbit corneas bears on this problem. A single injection produces transient edema, slight sudanophilia, no foam-cellular response, and no vascularization. Yet, as noted earlier, it is most probable that man experiences multiple episodes of vascular injury and subsequent increased permeability of the intimal endothelium and connective tissue to the lipid macromolecules in serum. So again the cornea model can be appropriately adapted by repeated injections of serum, this time of normal rabbit serum. Four injections of 0.3 ml. of serum with a cholesterol content of only 35 mg. % produces a small sudanophilic, foam-cellular lesion. Vascularization also occurs, and there are minimal changes in the collagen. Thus the corneal equivalent of the beginning of an atheroma is pro-

duced by four small injections of serum with a cholesterol content of only 35 mg. %, a total of only 0.42 mg. cholesterol in 1.2 ml. of serum. This morphologic sequence results probably because the water, electrolytes, and smaller proteins such as albumin in each 0.3 ml. of injected serum are removed from the avascular connective tissue while the large, comparatively less diffusible beta lipoglobulins remain and are concentrated. With each injection of serum the process repeats itself. The result is a concentration of lipid sufficient to elicit a lipophagocytic response and vascularization and to produce early collagen changes even though the lipid level in the initially injected serum is very low. There is definite evidence that concentration of lipid also occurs when hypercholesterolemic serum is repeatedly injected into the cornea, for in this case the Schultz test for cholesterol is strongly positive on corneal slices. A negative reaction is the rule in an equally swollen cornea after a single injection of hypercholesterolemic serum.

There is no reason to believe that this same mechanism of lipid concentration cannot or might not occur and progress to "fully developed" atherosclerotic plaques in human arteries in the presence of so-called normal or low serum lipid levels. All that need occur are a few brief periods of vascular injury productive of increased intimal permeability, and the atherosclerotic plaque is then well on its way to its final lethal role in the obstruction of a human coronary artery.

VI. SUMMARY

The pathogenesis of atherosclerosis in man is reviewed with emphasis on the lack of understanding in several important areas. The potential of the serum-injected rabbit cornea as an experimental model for pertinent reactions of the arterial intima is noted. Utilizing the corneal model, it has been possible to elucidate certain controversial mechanisms of both early and late stages of atherogenesis.

Thus it has been clearly demonstrated that after a single infiltration of avascular connective tissue by lipoprotein-rich serum, lipid appears initially as fine granules closely associated with collagen fibers. In only four days lipophagocytic cells appear. There is a steady progression of the lipid from an extra- to an intracellular location. During a full year of observation no necrosis of foam cells or release of lipid into the intercellular stroma could be seen. Over the course of a year there is a gradual decrease in the lipid content of the plaque, suggesting that the foam cells slowly metabolize the phagocytosed lipid. Microhemorrhages, as advancing capillary aneurysms burst, are a regular feature of neovascularization of corneal lipid-connective tissue plaques, and similar phenomenon may be the source of the larger hemorrhages in developing atheromata. By the removal of water from serum-infiltrated connective tissue, serum lipid is concentrated to such an extent that even four episodes of infiltration by lipid-poor serum, containing only 35 mg. % cholesterol, leads to the development of a fatty, foam-cellular lesion. This observation suggests

that concentration of serum lipids, and not *in situ* synthesis, may be responsible for the massive amounts of lipids regularly present in the aorta and coronary arteries in atherosclerosis.

VII. ILLUSTRATIONS

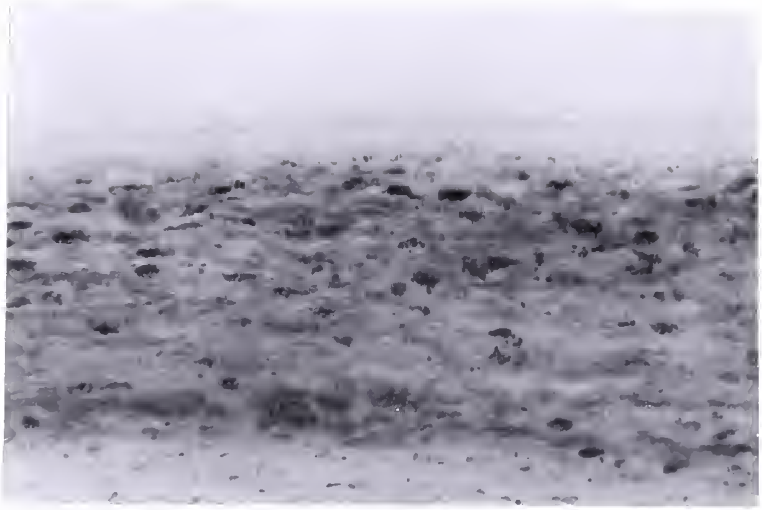

FIG. 1. Rabbit cornea six days after injection of 0.1 ml. of hypercholesterolemic rabbit serum. Lipid is both extracellular and within foam cells. (Sudan IV; orig. mag. X 100.)

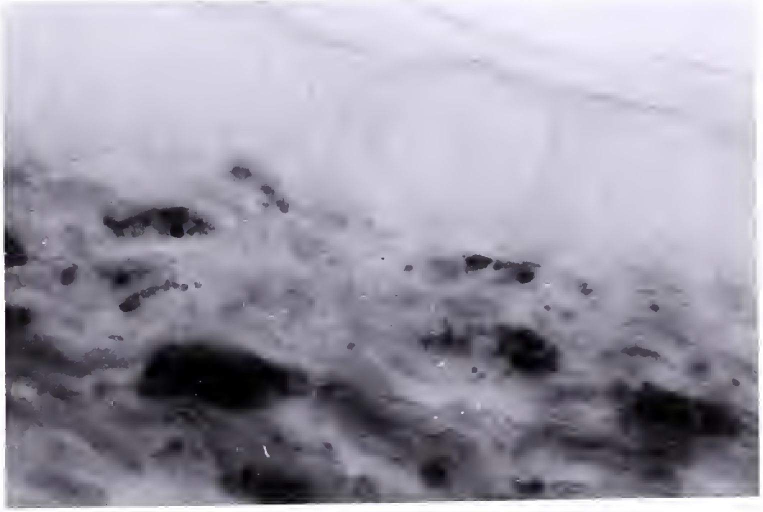

FIG. 2. Rabbit cornea six days after injection of 0.1 ml. of hypercholesterolemic rabbit serum. Lipid is present in two forms: 1) extracellular fine granules associated with collagen fibers; and 2) globules within foam cells. (Sudan IV; orig. mag. X 450.)

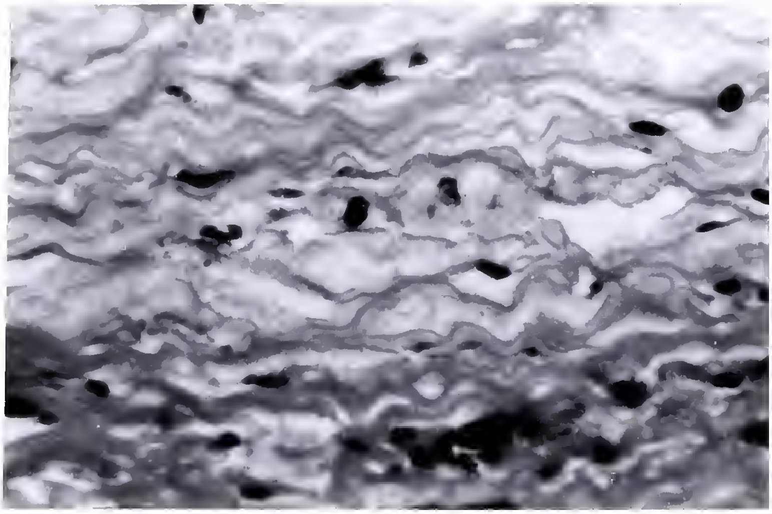

FIG. 3. Rabbit cornea one month after injection of 0.1 ml. of hypercholesterolemic rabbit serum. Foam cells are between frayed, fragmented collagen fibers. (H & E; orig. mag. X 450.)

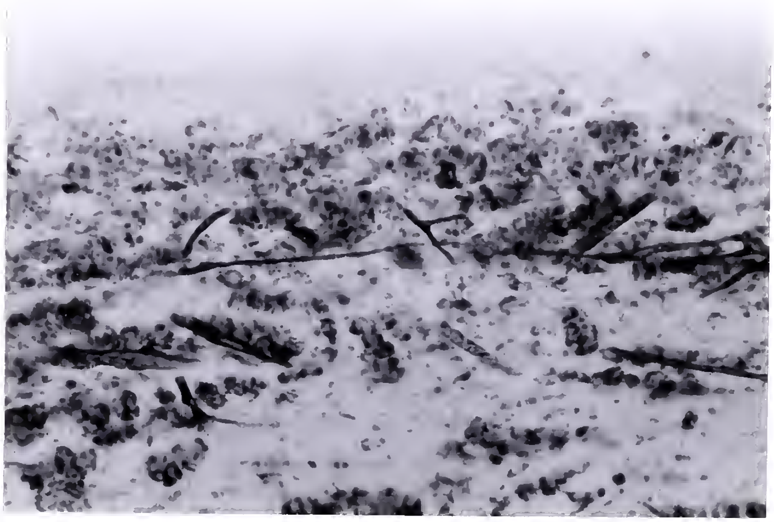

FIG. 4. Rabbit cornea one month after injection of 0.1 ml. of hypercholesterolemic rabbit serum. India ink-injected blood vessels are parallel to collagen lamellae and give off branches that cross these lamellae. Numerous foam cells are present. (Sudan IV; orig. mag. X 100.)

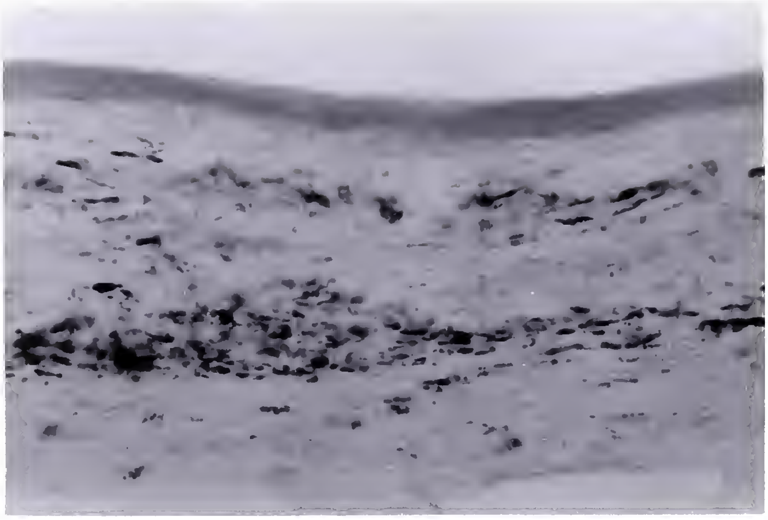

FIG. 5. Rabbit cornea one year after injection of 0.1 ml. of hypercholesterolemic rabbit serum. Foam cells are present. (Sudan IV; orig. mag. X 100.)

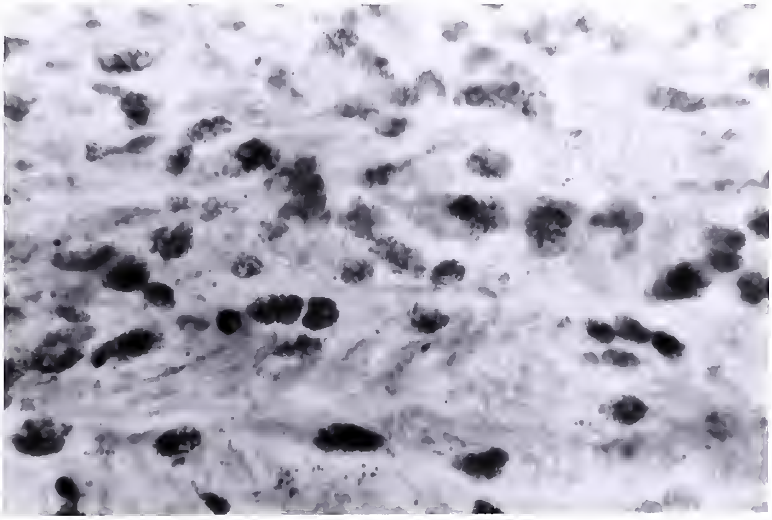

FIG. 6. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. There is a marked foam-cellular response. (Sudan IV; orig. mag. X 100.)

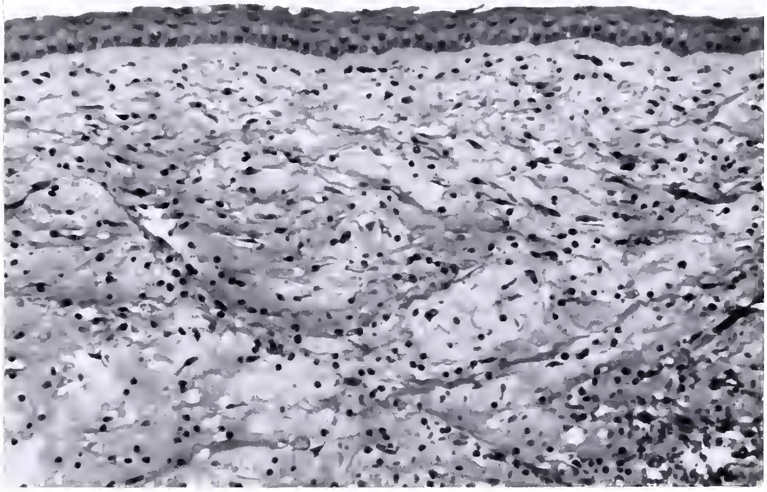

FIG. 7. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. There is a marked foam-cellular response in the very edematous plaque. (H & E; orig. mag. X 100.)

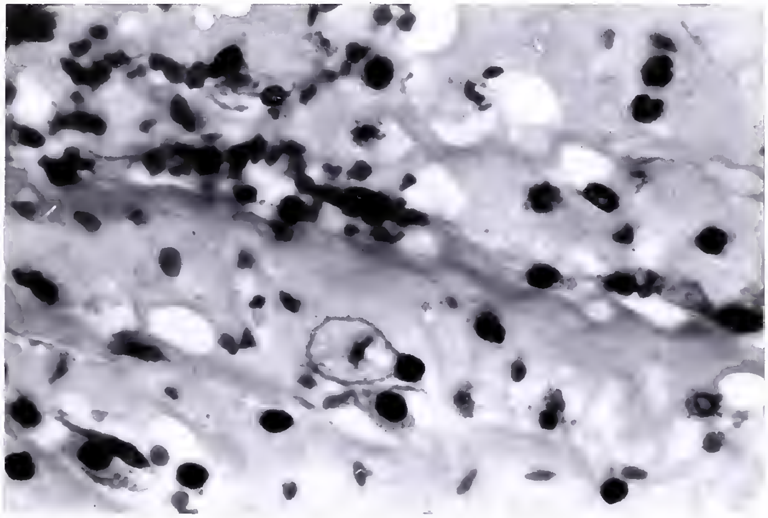

FIG. 8. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. Foam cells and an inflammatory cellular exudate are near the capillaries. (H & E; orig. mag. X 450.)

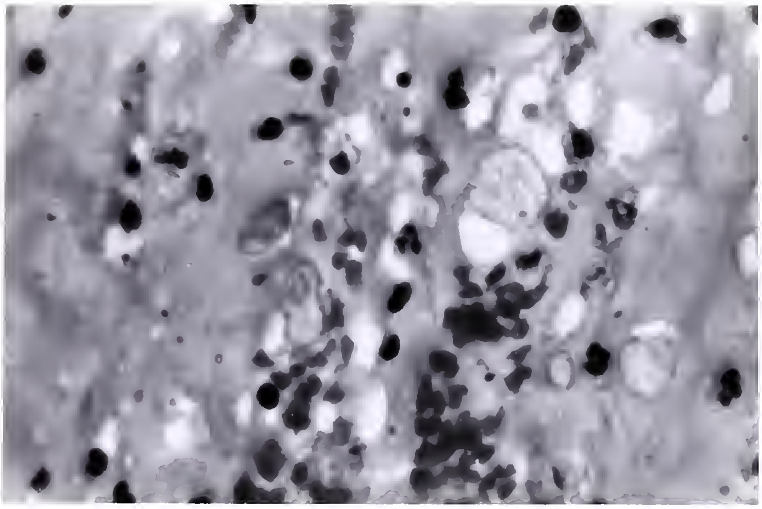

FIG. 9. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum three and two weeks before sacrifice. Dilated, congested capillaries are growing into the edematous plaque in which the collagen shows degenerative changes. Microhemorrhages are present at the advancing capillary tips. (H & E; orig. mag. X 450.)

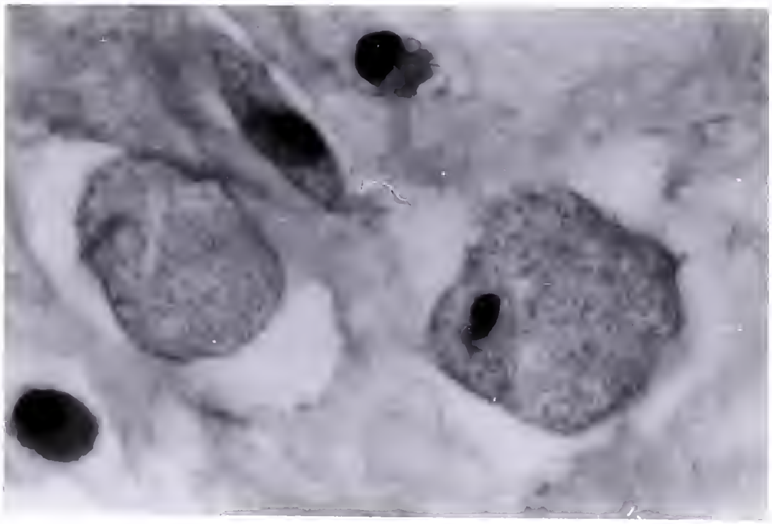

FIG. 10. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. Large foam cells of varying morphology are present in the extremely edematous plaque. (H & E; orig. mag. X 1,000.)

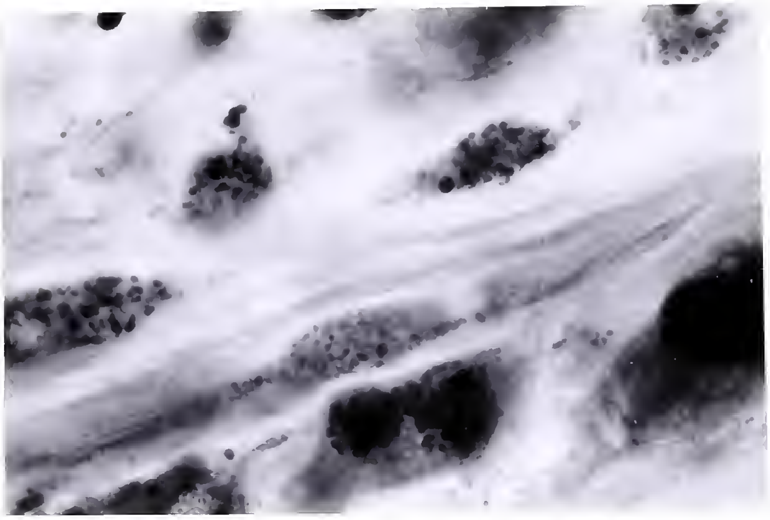

FIG. 11. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. Several large foam cells are between the lipid-impregnated collagen fibers. (Sudan IV; orig. mag. X450.)

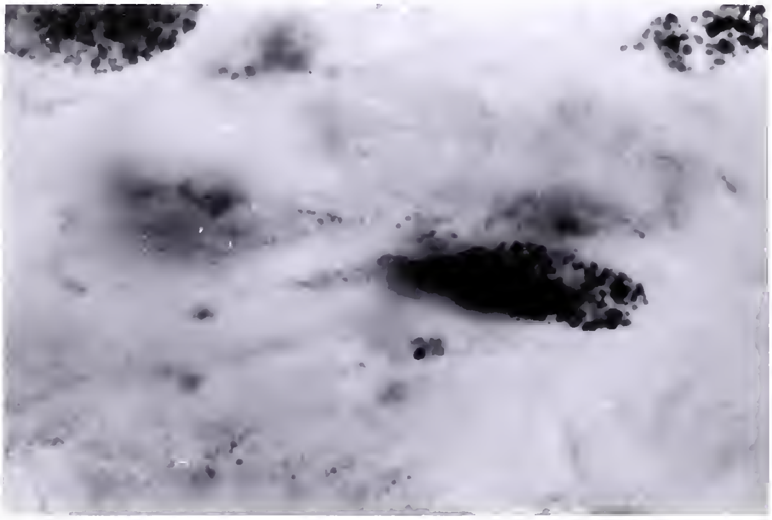

FIG. 12. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. An elongate foam cell with a slender projection of lipid-filled cytoplasm is seen. (Sudan IV; orig. mag. X 450.)

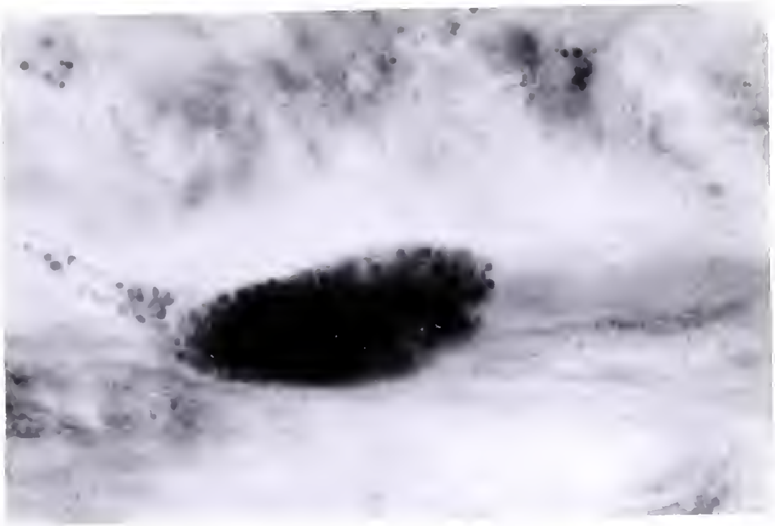

FIG. 13. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. An oval foam cell with a short projection of lipid-filled cytoplasm is seen. (Sudan IV; orig. mag. X450.)

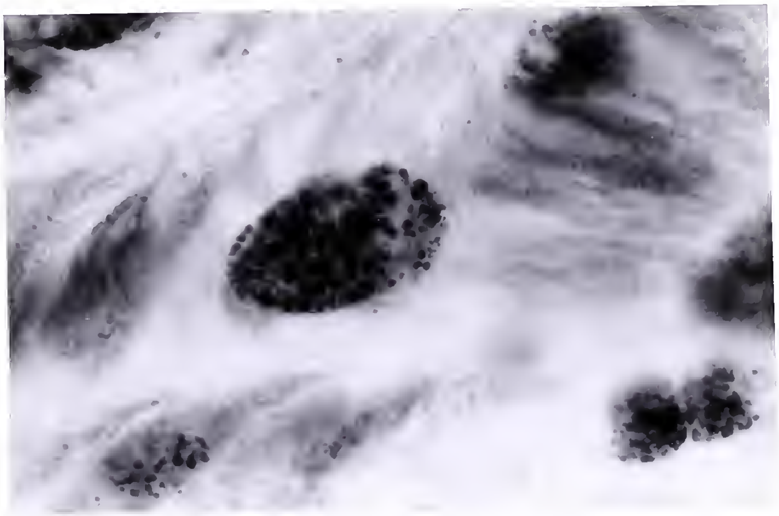

FIG. 14. Rabbit cornea three weeks after injection of 0.3 ml. of hypercholesterolemic rabbit serum. A large round foam cell is seen. (Sudan IV; orig. mag. X 450.)

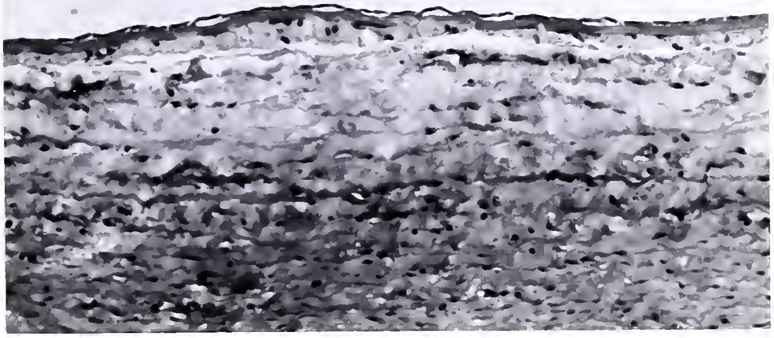

FIG. 15. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum three and two weeks before sacrifice. Foam cells are adjacent to Descemet's membrane. Marked edema and collagen changes are present. There is almost no cellular exudate. (H & E; orig. mag. X 100.)

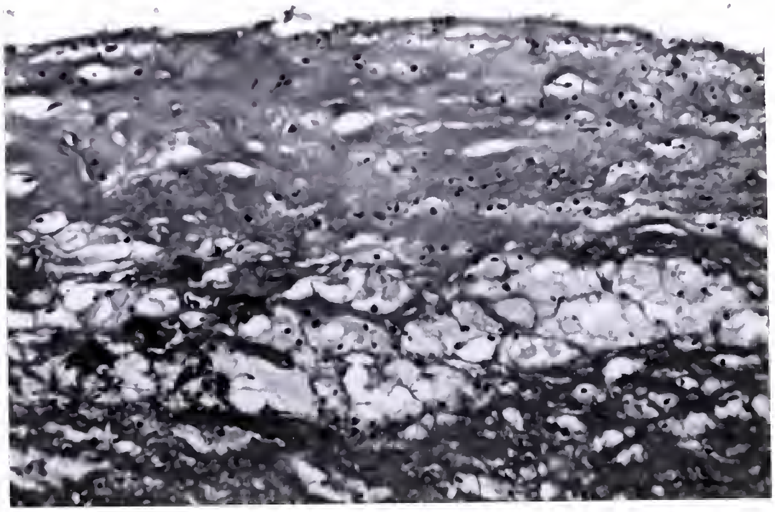

FIG. 16. Human aorta. Foam cells are in a region of swollen collagen. For comparison with Fig. 15. (H & E; orig. mag. X 100.)

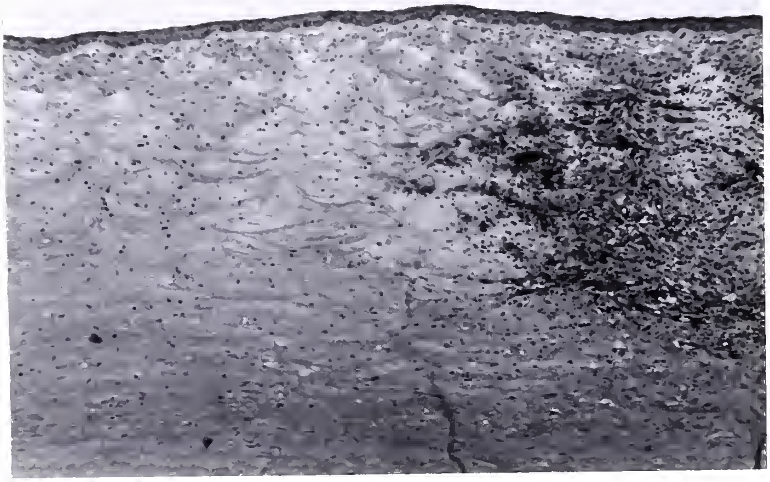

FIG. 17. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum $3\frac{1}{2}$, $2\frac{1}{2}$, and 2 weeks before sacrifice. Dilated, congested capillaries, surrounded by hemorrhages, are growing into the edematous, foam-cellular plaque. (H & E; orig. mag. X 40.)

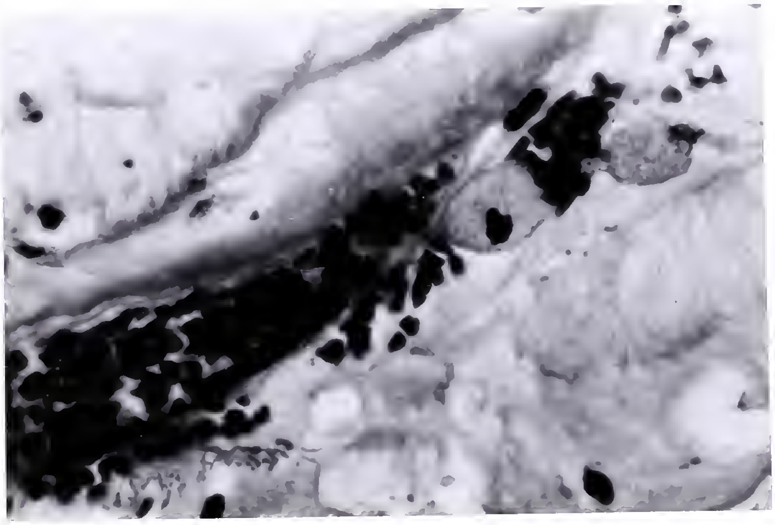

FIG. 18. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum $3\frac{1}{2}$, $2\frac{1}{2}$, and 2 weeks before sacrifice. Dilated, congested capillaries are growing into the edematous, foam-cellular plaque. There is a microhemorrhage at the advancing capillary tip. (H & E; orig. mag. X 450.)

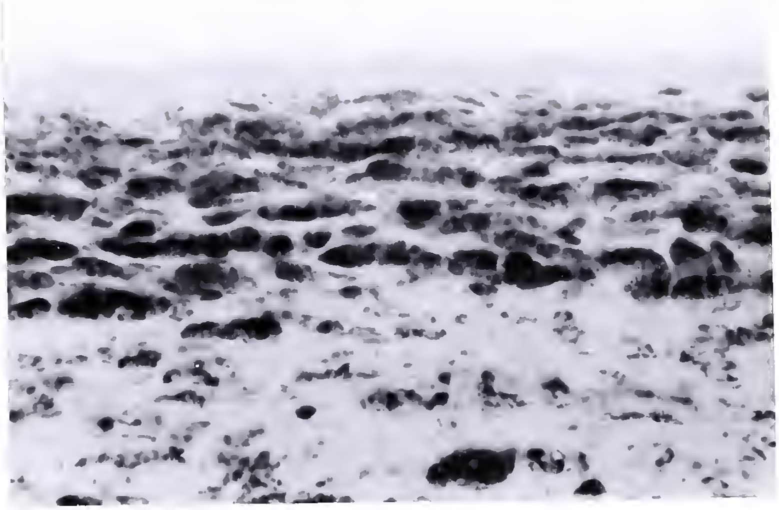

FIG. 19. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum 10, 6, and 5 weeks before sacrifice. An intensely foam-cellular lesion is present. (Sudan IV; orig. mag. X 100.)

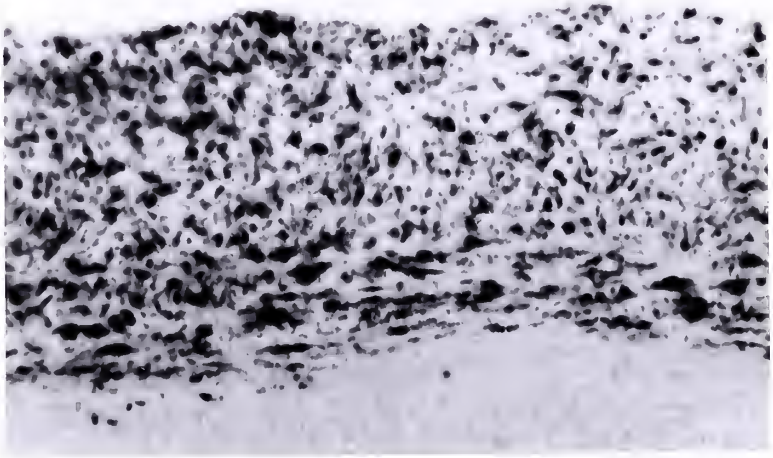

FIG. 20. Human aorta. An intensely foam-cellular reaction is seen in the thickened intima. For comparison with Fig. 19. (Sudan IV; orig. mag. X 100.)

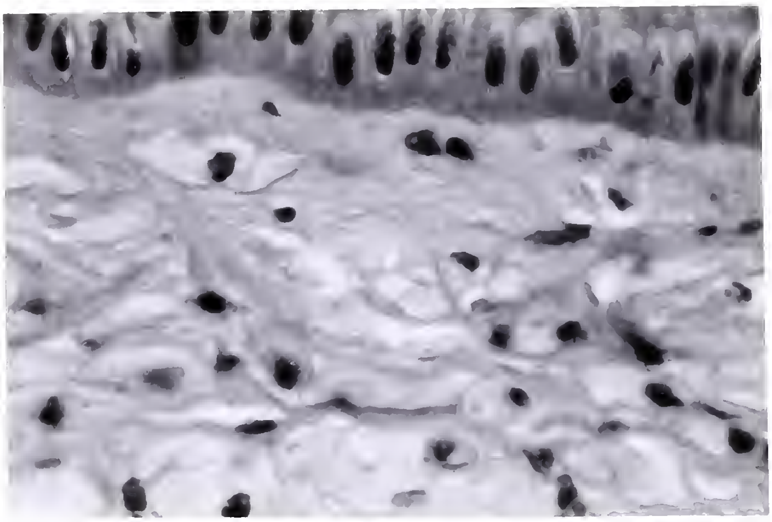

FIG. 21. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum 10, 6, and 5 weeks before sacrifice. Large foam cells are between the swollen, frayed collagen fibers beneath the corneal epithelium. (H & E; orig. mag. X 450.)

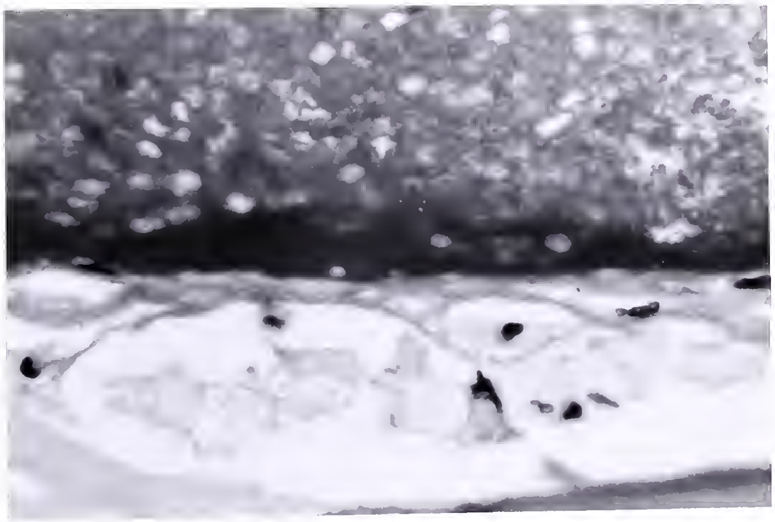

FIG. 22. Human coronary artery. Subendothelial foam cells are between the frayed collagen fibers. Blood is in the vessel lumen above. For comparison with Fig. 21. (H & E; orig. mag. X 450.)

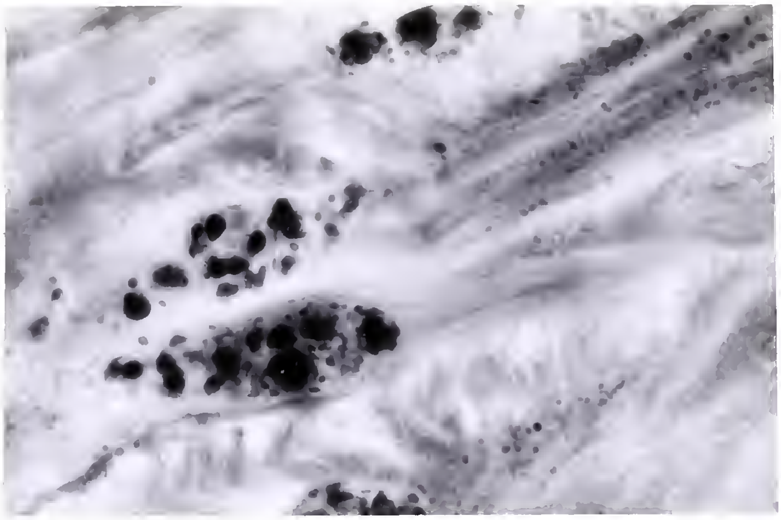

FIG. 23. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum 3½, 2½, and 2 weeks before sacrifice. Foam cells containing globules of lipid are between collagen fibers impregnated with finely granular, extracellular lipid. (Sudan IV; orig. mag. X 450.)

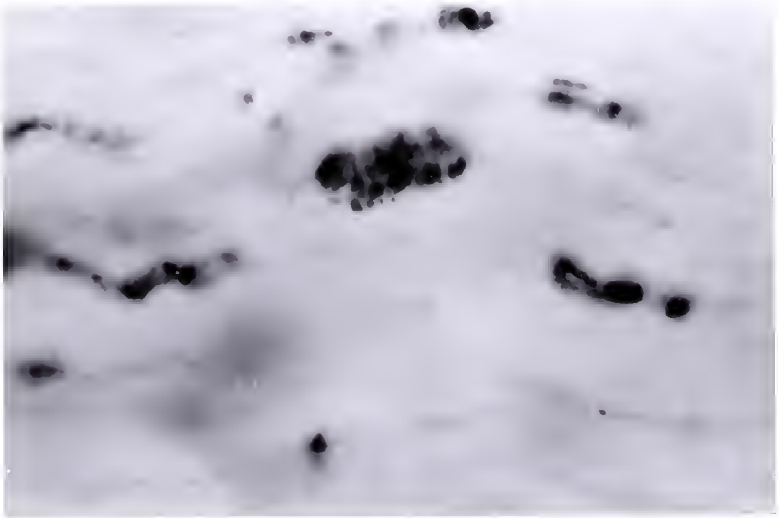

FIG. 24. Rabbit cornea injected with 0.3 ml. of <u>normal</u> rabbit serum (cholesterol 35 mg. %) four times at four-day intervals followed by two months without injections. Foam cells containing globules of lipid are seen. (Sudan IV; orig. mag. X 450.)

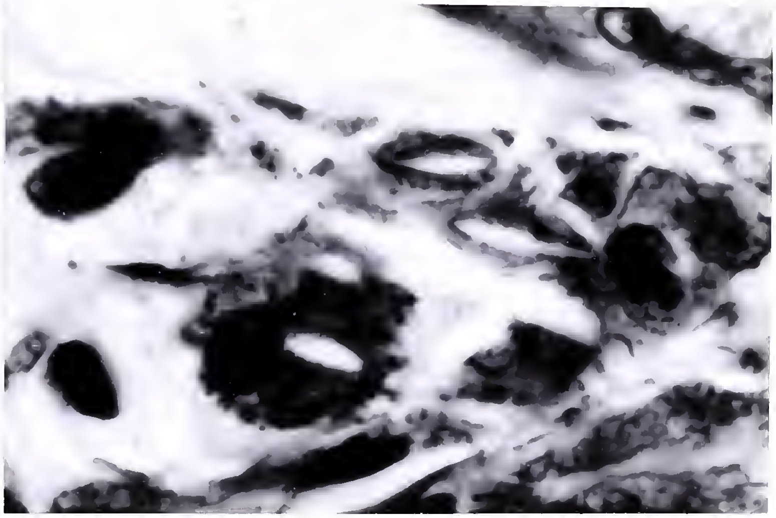

FIG. 25. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum five times at weekly intervals followed by two months without injections. Foam cells contain clefts of cholesterol ester crystals. (Masson; orig. mag. X 1,000.)

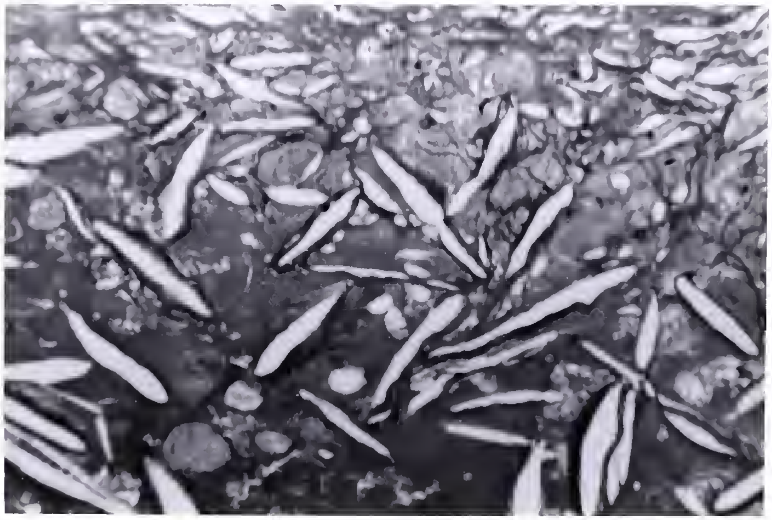

FIG. 26. Human aorta. Foam cells containing clefts of cholesterol ester crystals and similar but larger extracellular clefts are seen. For comparison with Fig. 25. (H & E; orig. mag. X 100.)

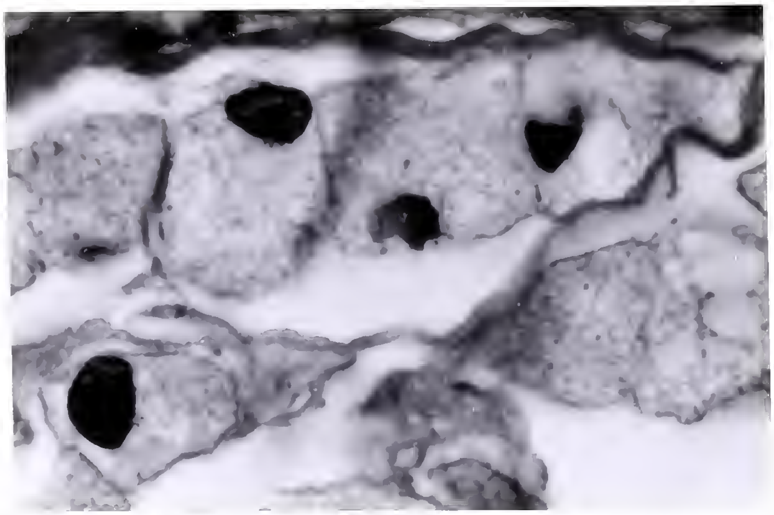

FIG. 27. Rabbit cornea injected with 0.3 ml. of hypercholesterolemic rabbit serum 10, 6, and 5 weeks before sacrifice. Foam cells are between the frayed collagen fibers. (H & E; orig. mag. X 1,000.)

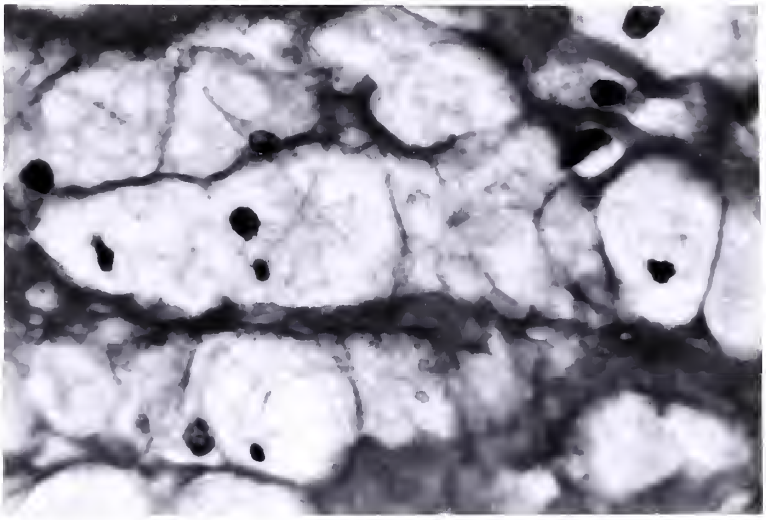

FIG. 28. Human aorta. Foam cells are between the frayed collagen fibers. For comparison with Fig. 27. (H & E; orig. mag. X 450.)

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